PHENACEIN — AN ANGIOTENSIN-CONVERTING ENZYME INHIBITOR PRODUCED BY A STREPTOMYCETE

I. TAXONOMY, FERMENTATION AND BIOLOGICAL PROPERTIES

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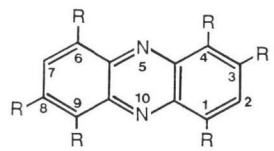
Phenacein, 3,6-dihydroxy-1-phenazinecarboxylic acid, was a specific angiotensin-converting enzyme (ACE) inhibitor isolated from a member of the *Streptomyces tanashiensis-zaomyceticus* group. Phenacein acted as a pure competitive inhibitor with a *Ki* of 0.58 μM. ACE inhibition could be reversed by Zn^{++}, but not by Co^{++}, Ca^{++}, or Mg^{++}; therefore, phenacein may chelate the active site zinc of ACE. However, other zinc-containing enzymes were not inhibited at high phenacein concentrations. Phenacein exhibited weak activity against Gram-positive bacteria, but was not active against *Candida* sp. or Gram-negative organisms.

Angiotensin-converting enzyme (ACE) is an important target for the treatment of hypertension. Clinically effective ACE inhibitors include the peptide analog captopril[1] and teprotide[2], a nonapeptide isolated from the venom of *Bothrops jararaca*. Several novel ACE inhibitors from microorganisms have recently been described. These include ancovenin[3], an unusual peptide containing sixteen amino acids, isolated from an actinomycete; the aspergillomarasmines[4], pseudopeptides produced by a variety of fungi; and the muraceins[5,6], a family of muramyl peptides isolated from *Nocardia orientalis*.

In our screening program designed to identify naturally occurring ACE inhibitors from microbial sources, 3,6-dihydroxy-1-phenazinecarboxylic acid (Fig. 1) was identified as a specific inhibitor of ACE. Taxonomy of the producing organism, fermentation and biological properties of phenacein* are discussed in this paper. Isolation and structure determination are described in the following paper[7].

Fig. 1. Structures of phenazine-containing molecules.



Compound	R_1	R_3	R_4	R_6	R_8	R_9
Phenacein	COOH	OH	H	OH	H	H
SQ 28,232	COOH	H	H	OH	H	H
SQ 28,102	COOH	OCH_3	H	OCH_3	H	H
Lomofungin	OH	H	$COOCH_3$	OH	OH	CHO
Phenazine	H	H	H	H	H	H

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* Nomenclature is derived from the words PHENazine ACE INhibitor.

Taxonomy

Culture SC 12730 (ATCC 39460) was identified as a *Streptomyces* sp. belonging to the *Streptomyces tanashiensis-zaomyceticus* group based on procedures employed in the International Streptomyces Project[8].

SC 12730 produced both vegetative and aerial mycelium. Spores were borne in straight chains (*Rectus flexibilis* group). The spore surface was smooth as seen by transmission electron microscopy. The spore color was gray on all media supporting sporulation. The reverse color was brownish gray on oatmeal agar (ISP-3). Melanoid pigment was produced on tyrosine-containing media (ISP-1 and 7). Glucose, xylose and arabinose were utilized as sole carbon sources, whereas rhamnose, fructose, raffinose, mannitol, inositol and sucrose were not.

Materials and Methods

Reagents

Captopril, teprotide, SQ 28,232[9], SQ 28,102[7], and the ACE substrates furanacryloylphenylalanylglycylglycine (FAPGG)[10] and *p*-nitrobenzyloxycarbonylglycyl-(*S*-4-nitrobenzo-2-oxa-1,3-diazole)-L-cysteinylglycine (NBGCG)[11] were synthesized at the Squibb Institute. Phenazine was from Sigma Chemical Company, St. Louis, Missouri, and lomofungin was obtained from Upjohn, Kalamazoo, Michigan.

Enzyme Preparation

Rabbit lung ACE was homogenized and chromatographed on Sephadex G-75 as described previously[5].

ACE Assays

Assays were performed spectrophotometrically by following hydrolysis of either FAPGG at 25°C or NBGCG at 37°C in 50 mM Tris-HCl containing 0.3 M NaCl, pH 7.5, as described by BUSH *et al.*[5]. *Ki* determinations were calculated using linear regression analysis of data plotted according to LINEWEAVER-BURK and DIXON[12]. Effects of ACE inhibitors on smooth muscle contraction were tested in excised guinea pig ileum using the method of RUBIN and collaborators[13,14].

Effects of divalent metal ions were determined as follows: In one study 800 μl NBGCG (100 μM) and 5 μl phenacein (0.32 mM) were mixed with 25 μl ACE. Initial rates of reaction were determined for 3~4 minutes before 2~10 μl of $ZnSO_4$ (1~100 mM) was added. The change in absorbance was then followed for another 8~10 minutes. In other experiments 15 μl of ACE and 20 μl phenacein (0.48 mM) were preincubated 5.0 minutes to allow equilibration of the enzyme-inhibitor complex; 10 μl of 0.10 M $ZnCl_2$ was then added at 25°C. After 2.0 minutes 900 μl FAPGG (1.25 mM) was added and the change in absorbance monitored. The latter set of experiments was repeated using $CoCl_2$, $MgCl_2$ and $CaCl_2$. In all studies, controls were run containing enzyme, buffer, M^{++}, and substrate, as divalent cations may react allosterically to alter ACE activity (K. BUSH, unpublished data).

Fermentation

Seed cultures of *Streptomyces* sp. SC 12730 were prepared by transferring a loopful of surface growth from an agar slant into 500-ml Erlenmeyer flasks containing 100 ml of the following medium: 0.40% yeast extract, 1.0% malt extract and 0.40% dextrose in distilled water. The pH was adjusted to 7.3 before sterilization. The flasks were then incubated at 25°C on a rotary shaker (300 rpm, 5-cm stroke) for approximately 72 hours.

A 5% (v/v) transfer was made from the seed culture flasks to 500-ml Erlenmeyer flasks containing 100 ml each of the following: Citric acid 1.28%, $(NH_4)_2SO_4$ 0.60%, $MgSO_4 \cdot 7H_2O$ 0.025%, KH_2PO_4 0.015%, $MnSO_4 \cdot 4H_2O$ 0.001% and $CaCO_3$ 1.1% in distilled water. At the time of inoculation 5.0 ml of sterile glucose (20%) was added to each flask. The flasks were then incubated at 25°C on a rotary shaker (300 rpm, 5-cm stroke) for 120 hours. At harvest the flask contents were pooled. After centrifugation, the resulting supernatant was used for the isolation of phenacein.

Results and Discussion

Phenacein inhibited ACE with an I_{50} value similar to that of teprotide (Table 1). When compared to other phenazine-containing molecules, phenacein was the most active ACE inhibitor in the series studied. Mechanistic studies indicated that phenacein behaved like a pure competitive inhibitor (Fig. 2) with a *Ki* of 0.58 μM.

Chelation of the ACE active site zinc is a possible explanation for the inhibitory activity of molecules such as the aspergillomarasmines[4]. Thus, the effects of divalent cations on phenacein inhibition were studied. Reversal of ACE inhibition by phenacein could be accomplished in the presence of $ZnSO_4$ or $ZnCl_2$, utilizing either FAPGG or NBGCG as substrate. In one set of experiments 1.9 μM phenacein inhibited ACE activity 85%. When $ZnSO_4$ was added to the reaction mixture containing enzyme, inhibitor and NBGCG as substrate, the reaction rate immediately increased. Partial reversal of inhibition was observed at concentrations as low as 10 μM Zn^{++}, with a maximal effect (90% control activity) at 1.5~2.0 mM Zn^{++}. In other studies with FAPGG as substrate, reversal plateaued at 1.0 mM Zn^{++}. The order of addition of Zn^{++}, either before or after substrate, did not affect reversal characteristics. Phenacein inhibition of ACE, however, could not be reversed by 1.0 mM $CoCl_2$, $MgCl_2$ or $CaCl_2$. This behavior was in contrast to that of aspergillomarasmine A, where ACE inhibition was completely reversed by stoichiometric additions of $ZnCl_2$ or $CoCl_2$ (K. BUSH and P. HENRY, unpublished data).

Because phenacein appeared to exhibit specificity for chelation of Zn^{++}, this inhibitor was tested for its effects on other enzymes that contain zinc at the active site. At a phenacein concentration of 20 μM, liver alcohol dehydrogenase and carboxypeptidase A were inhibited less than 5%; at an inhibitor concentration of 80 μM, carboxypepetidase B activity decreased 11%. Thus, phenacein was highly selective for inhibition of ACE. When phenacein was tested for its effect on smooth-muscle contracting activity in the excised guinea pig ileum, the EC_{50}* for angiotensin I antagonism was 24 μM; the EC_{50} for bradykinin potentiation was 1.2 μM. The activity profile generated from these

Table 1. I_{50} values of selected ACE inhibitors.

Rabbit lung ACE (10 μl) and inhibitor or buffer (100 μl) were incubated 5 minutes at 37°C before NBGCG (1,000 μl) was added to initiate reaction.

Hydrolysis rates were monitored at 37°C.

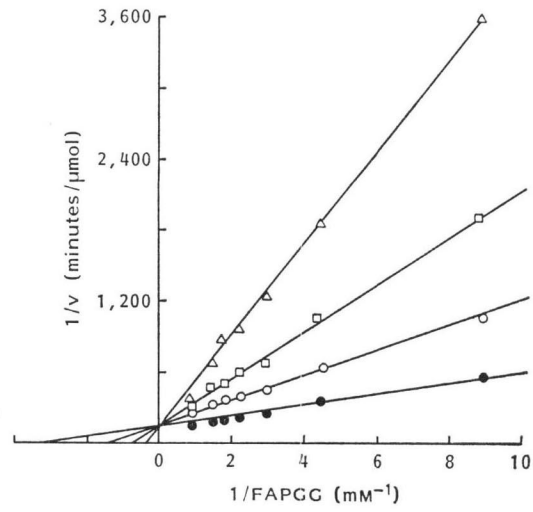
Inhibitor	I_{50} (nM)
Captopril[a]	0.65
Teprotide[b]	130
Phenacein	390
SQ 28,232	920
SQ 28,102	>500,000
Phenazine	>500,000
Lomofungin	16,000

[a] D-3-Mercapto-2-methylpropanoyl-L-proline.
[b] <Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro

Fig. 2. LINEWEAVER-BURK plot of inhibition of ACE by phenacein.

ACE and inhibitor or buffer were incubated 5 minutes in a volume of 115 μl at 25°C before the addition of 1.0 ml FAPGG (0.11~1.1 mM).

Phenacein concentrations: ●, 0 μM; ○, 0.66 μM; □, 2.0 μM; △, 6.6 μM.



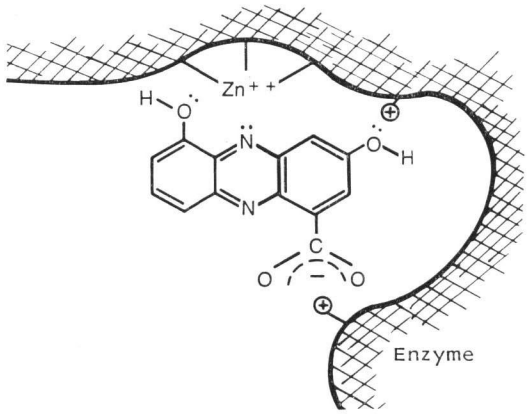
* Effective concentration producing a 50% change in agonist effect.

Table 2. Antimicrobial activity of phenacein against representative organisms.

Organism	MIC (μg/ml)
Candida albicans SC 5314	>100
C. albicans SC 11422	>100
Staphylococcus aureus SC 1276	50
Streptococcus faecalis SC 9011	1.6
S. agalactiae SC 9287	50
Micrococcus luteus SC 2495	6.3
Escherichia coli SC 8294	>100
Pseudomonas aeruginosa SC 9545	>100

All organisms were tested on F4 agar using serial dilutions. *Candida* sp. were at 10^3 cfu, bacteria were at 10^4 cfu.

Fig. 3. Possible interaction of phenacein with ACE.



studies supported the observation that phenacein was a specific ACE inhibitor.

Phenacein at levels of 100 μg/ml exhibited no antifungal activity when tested against twelve strains of *Candida albicans*. However, weak antibacterial activity was observed against selected Gram-positive organisms (Table 2).

Phenacein was a specific ACE inhibitor that exhibited the potential for bidentate chelation of Zn^{++}. It may be postulated that the active site zinc of ACE could be complexed by the electron-rich 5-nitrogen and 6-hydroxyl functionalities of the phenacein as shown in Fig. 3. The I_{50} results support this type of interaction, as the best phenazine-containing inhibitors were phenacein, SQ 28,232 and lomofungin, all of which contain 6-hydroxyl groups.

Because ACE was inhibited more potently than other zinc-containing enzymes, enzyme-inhibitor interactions other than simple complexation of the active site zinc must occur. The activities of phenacein and SQ 28,232 suggest that a second interaction with the enzyme may, therefore, involve the 1-carboxyl moiety. This anionic moiety may interact with a cationic group at the active site, possibly binding at the same site as the carboxy terminus of natural ACE substrates. A minor role for involvement of the 3-hydroxyl is suggested, as loss of this functional group in SQ 28,232 resulted in less than a three-fold increase in I_{50}. Overall, chelation of the ACE zinc by the 6-hydroxyl group must be most critical in determining inhibitory activity, as indicated by the complete lack of inhibition by the 3,6-dimethylated derivative, SQ 28,102 which still retained the 1-carboxyl.

It is interesting to compare the properties of phenacein with lomofungin. Lomofungin, a penta-substituted phenazine antibiotic isolated from *Streptomyces*, is active against bacteria, yeasts and filamentous fungi[15], whereas phenacein was only weakly antibacterial and exhibited no anticandidal activity. Lomofungin is postulated to work by chelating the bound zinc of RNA polymerase[16]. However, it was only weakly active against ACE. These results indicate that the specificity of ACE inhibition observed with phenacein is due to interactions other than simple chelation of zinc.

The observed specificity for inhibition is noteworthy for several of the microbially-produced ACE inhibitors such as phenacein, muracein A[5], and the aspergillomarasmines[4]. However, I_{50} values for these inhibitors are in the range of $0.3 \sim 1.2$ μM. Another streptomycete-produced ACE inhibitor, related to marasmine, L-681,176, has an I_{50} value in the same range (3.7 μM).[17] These values are all at least two orders of magnitude higher than values observed for captopril in the same assays. Thus,

further synthetic modifications would be necessary before any of these naturally occurring inhibitors could be utilized clinically as a viable drug candidate for the treatment of hypertension.

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